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Enhancement of metabolic rates of yeast flocculent cells through the use of polymeric additives

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Abstract. The influence of several polymeric additives on specific glucose uptake rate of flocs of a S. cerevisiae strain – S. cerevisiae NRRL Y 265 was studied. A special continuous membrane microreactor was used to measure glucose uptake on the presence of calcium and of the tested additives – two cationic polymers – bis(polyoxyethylene-bis(amine)) 20,000 and BPA 1,000 and one anionic polymer – Magna Floc LT25.

An increase on glucose uptake rate was always observed when comparing with calcium bound flocs. For bis(polyoxyethylene-bis(amine)) 20,000 the increase was only 19% but for BPA 1,000 a value of more than 50% was observed. For Magna Floc LT25 a two fold increase was measured.

The determination of floc size and porosity in the presence of the additives indicated that, on the basis of these parameters, it was not possible to explain the observed glucose uptake rates. The floc porosites in additive bound flocs were similar and 10% larger than for calcium bound flocs and glucose uptake rate was larger for the largest flocs – Magna Floc LT25 bound flocs were the largest followed by BPA 1,000, bis(polyoxyethylene-bis(amine)) 20,000 and calcium bound flocs. These values disagree with what should be expected in diffusion controlled processes.

The calculation of intercellular floc distance indicated that polymeric additives act on the reduction of diffusional limitations by increasing the available flux area for glucose inside the flocs. By analysing different kinds of packings, it was also observed that the packing arrangement for yeast cells in flocs is close to the cubic packing. The simulation of this arrangement for the obtained floc sizes confirmed that the 10% increase in floc porosity is sufficient to explain the increase in the available flux area.

1 Introduction

One of the main advantages of the systems that use flocculating microorganisms – bacteria or yeast – is the possibility of obtaining high cell density concentrations, thereby increasing the volumetric conversion rate of the reactor [1–8].

However, microbial aggregates are characterized by relatively low specific reaction rates. Nutrients have to reach the cells inside the flocs by diffusing into the floc particles [9–13].

The diffusion of a solute into the floc particles, expressed as effective diffusivity is a function of:

- solute diffusivity in the medium
- floc size
- floc porosity
- floc tortuosity

In most cases, the substrate diffusion rate towards the intrafloc cells is lower than the cellular metabolic rate and therefore substrate cannot be metabolized at the highest possible rate. In such cases, it is said that the overall reaction is mass transfer limited rather than biochemically limited. In order to obtain maximum productivity, mass transfer limitations should be minimized.

Adjacent cells of flocculent strains are linked to each other through bridges where calcium ions play an important role [14–18]. One possible way to circumvent mass transfer limitations could be the use of additives that may act as bridge extenders, thereby enlarging the accessible space between cells [19].

In this work, the measurement of specific glucose uptake rate in a continuous microreactor allowed for the analysis of the influence of several additives in the reduction of diffusional limitations in a flocculating strain of S. cerevisiae – S. cerevisiae NRRL Y 265.

Simultaneous floc size analysis and intrafloc void volume determinations were made to try to correlate the change in glucose uptake rate with the physical characteristics of the flocs that control effective glucose diffusivity.

2 Materials and methods

2.1 Materials

Two strains of Saccharomyces cerevisiae were used:

- flocculating one – S. cerevisiae NRRL Y 265
- a non flocculating one – S. cerevisiae sake

The medium, per cubic meter of tap water, was composed of

KH_2PO_4	5 kg
$(NH_4)_2SO_4$	2 kg
$MgSO_4 \cdot 7H_2O$	0.4 kg
Yeast extract	1 kg

The concentration of calcium chloride, when used, was $1 \cdot 10^{-3}$ M.

The carbon source was glucose, whose initial concentration was 50 kg m^{-3} for yeast growth and 5 kg m^{-3} on the experiments for the determination of specific glucose uptake rate. The pH was, in all experiments, adjusted to 4.0 ± 0.1 by the addition of H_3PO_4. The temperature was controlled at 30 °C. For yeast growth, medium was autoclaved at 121 °C during 30 minutes.

2.2 Cell preparation

Cells of both strains were grown for 24 hours in 1 liter Erlenmeyer flasks containing the above described medium. Then they were harvested by centrifugation. The flocculent cells were deflocculated by washing three times with a NaCl 15 kg m^{-3} solution at pH 2, after that they were washed three times with ultrapure water. Flocculent cells were then ready to be incubated in the 5 kg m^{-3} glucose medium with the desired additive.

Such treated cells were used for measuring floc size, floc porosity and glucose specific uptake rate.

S. cerevisiae sake cells were only washed three times with ultrapure water before being added to the desired medium.

2.3 Analytical

Glucose concentration was measured by the DNS method [20].

Biomass concentration was determined by measuring biomass dry weight [21].

2.4 Additives

The charged polymers added to the medium were:

- Bis(polyoxyethylene-bis(amine)) 20,000 – (P 20,000)
- BPA 1,000
- Magna Floc LT25

Bis(polyoxyethylene-bis(amine)) 20,000 is a cationic tetravalent polyethylene glycol derivative with a molecular weight of 20,000 (obtained from SIGMA Co.). BPA 1,000 is a cationic polymer (quaternary amine) of styrene-divinylbenzene (obtained from ROHM & HASS Co.). Magna Floc LT25 is a high molecular weight anionic polyacrylamide (obtained from ALLIED COLLOIDS Ltd.).

In all experiments the additives concentration was 0.01% (w/v).

2.5 Determination of sedimentation capacity

The flocculation capacity of S. cerevisiae flocculating strain was assayed using a modification of the Helm sedimentation test [15].

After preparing the cells as previously described the sedimentation capacity was measured as follows:

The cells were suspended in a $25 \cdot 10^{-6}$ m^3 beaker containing a $1 \cdot 10^{-3}$ M $CaCl_2$ ultrapure water solution and 0.01% of the desired additive. At defined intervals, samples were taken from a fixed position in the beaker (level corresponding to $15 \cdot 10^{-6}$ m^3).

The normalized cell concentration, defined as the ratio between actual and initial cell concentration was plotted against sedimentation time. A sedimentation profile was obtained.

Initial cell dry weight concentration in the beaker was 4 kg m^{-3}.

2.6 Measurement of specific glucose uptake rate

The determination of this parameter was made in a specially designed continuous microreactor (Fig. 1). It consisted of a 0.05 m thick plexiglass system formed by two chambers separated by a $0.45 \cdot 10^{-6}$ m membrane filter. The lower chamber had a volume of $40.45 \cdot 10^{-6}$ m^3 and was stirred with a magnetic bar. The volume of the upper chamber was $4.81 \cdot 10^{-6}$ m^3.

The cells were resuspended with the medium containing the desired additive and placed in the lower chamber of the microreactor. After completely filling this chamber with this medium, the system was sealed and feeding started. Fresh medium with the additive was fed to the microreactor by a peristaltic pump at a dilution rate of 2 h^{-1}. Samples were collected from the outflow at defined intervals to determinate glucose consumption. The cell dry weight was determined after each experiment.

2.7 Floc size analysis

Floc analysis was done by wet sieving the flocs through sieves of standard size.

Flocs treated as described in a previous section were placed in contact with the medium that contained the desired additive inside a $250 \cdot 10^{-6}$ m^3 flask and stirred for 15 minutes at the same speed that was used for the determination of the kinetic parameters.

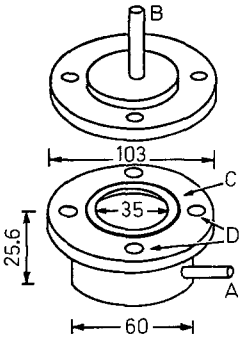

Fig. 1. Exploded view of the continuous microreactor. A – Feed inlet; B – Outlet; C – O-ring; D – Butterfly screw valves (all dimensions are expressed in 10^{-6} m)

The content of the flask was then poured in the sieving device: a group of six standard size sieves – range $37 \cdot 10^{-6}$ m–$180 \cdot 10^{-6}$ m. This system was vibrated for 5 minutes at a low speed in order to minimize floc destruction.

2.8 Floc porosity analysis

The porosity, defined as the ratio between intrafloc water mass and total floc mass, was determined with a thermogravimetric balance (0.01 mg precision) using the procedure described by Uribelarrea [22].

2.9 Floc specific gravity analysis

Floc specific gravity determination was done using a $25 \cdot 10^{-6}$ m^3 picnometer. In this technique [9], the water mass (M0) displaced by a known flocs mass (M1) was measured. Flocs specific gravity is the ratio M1/M0. The flocs used in this procedure were treated as for floc porosity analysis.

3 Results and discussion

When testing the influence of flocculation additives on glucose consumption rate, our initial purpose was to check whether these additives would change the sedimentation capacity of the flocs.

The results obtained with the sedimentation tests (Fig. 2) clearly showed that the flocculation ability of S. cerevisiae NRRL Y 265 flocs was not changed by the presence of the tested additives. The obtained sedimentation profiles were the same in every case.

After confirming the maintenance of the flocculation capacity of the yeast flocs, the measurement of specific glucose uptake rate was made.

These measurements were made at a dilution rate of 2 h^{-1} and during a short period (30–40 minutes). With these experimental conditions it was assumed that no cell growth would occur during the experiments. Even assuming a maximum specific growth rate as high as 0.4 h^{-1}, the cells would have a minimum doubling time of 1.7 h which validates the former assumption.

In all experiments, the agitation speed of the magnetic bar was 200 r.p.m., assessed by a stroboscopic tachometer. This speed, as previously shown [13], allowed for the elimination of external diffusional limitations in the flocs.

Considering biomass concentration X (kg m^{-3}), expressed as cell dry weight, and S (kg m^{-3}) the glucose concentration in the medium at time t, the specific glucose consumption rate is defined as:

$$+q_S = -1/X \cdot dS/dt \quad (1)$$

and so

$$1/X \cdot dS = -q_S \cdot dt \quad (2)$$

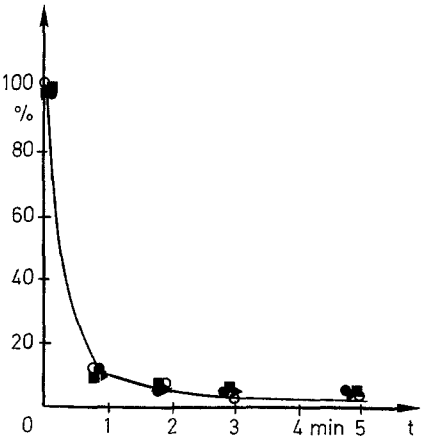

Fig. 2. Floc sedimentation profile for the tested additives. ● – Ca^{2+}; ▸ – bis(polyoxyethylene-bis(amine)) 20,000; ■ – BPA 1,000; ○ – Magna Floc LT25

Table 1. Operating conditions and results for the diffusion experiments for the flocculating strain

Additive	Initial glucose conc. (kg m^{-3})	Dry weight (kg)	$-q_S$ (kg kg^{-1} · s^{-1})	$-q_S$ (av)	Correlation coefficient
Ca^{2+}	5.81	0.342	$6.00 \cdot 10^{-5}$		0.999
				$5.92 \cdot 10^{-5}$ $\pm 1.3 \cdot 10^{-6}$	
	5.04	0.450	$5.82 \cdot 10^{-5}$		0.999
P 20,000	4.98	0.383	$6.80 \cdot 10^{-5}$		0.999
				$7.05 \cdot 10^{-5}$ $\pm 3.3 \cdot 10^{-6}$	
	5.62	0.174	$7.28 \cdot 10^{-5}$		0.998
BPA 1,000	5.67	0.152	$8.50 \cdot 10^{-5}$		0.999
				$8.90 \cdot 10^{-5}$ $\pm 5.3 \cdot 10^{-6}$	
	4.58	0.286	$9.23 \cdot 10^{-5}$		0.999
Magna Floc LT25	5.48	0.146	$11.38 \cdot 10^{-5}$		0.997
				$11.67 \cdot 10^{-5}$ $\pm 3.3 \cdot 10^{-6}$	
	5.54	0.142	$11.87 \cdot 10^{-5}$		0.999

Thus, q_S can be calculated by the slope of the straight line obtained by plotting S/X against time. Table 1 displays the conditions of each experiment as well as the values obtained for the specific substrate consumption rate and the correlation coefficients for each straight line.

It is clear, from these results, that the presence of the additive changed the glucose assimilation ability of the tested strain S. cerevisiae NRRL Y 265. The additive bis(polyoxyethylene-bis(amine)) 20,000 had the smallest effect and only a 19% increase on specific glucose uptake rate was observed. On the other hand, there was a clear difference between the slope of the calcium straight line and the slopes of the BPA 1,000 and Magna Floc LT25 straight lines. For BPA 1,000 the increase on specific glucose uptake rate was 50% and for Magna Floc LT25 there was a 2 fold increase in this parameter.

Table 2. Operating conditions and results for the diffusion experiments for the non flocculating strain

Additive	Initial glucose conc. ($kg\,m^{-3}$)	Dry weight (kg)	$-q_S$ ($kg\,kg^{-1}\cdot s^{-1}$)	$-q_S$ (av)	Correlation coefficient
Ca^{2+}	5.78	0.533	$14.37\cdot10^{-5}$	$14.57\cdot10^{-5}\pm1.3\cdot10^{-6}$	0.998
	5.78	0.386	$14.77\cdot10^{-5}$		0.999
Magna Floc LT25	5.22	0.528	$15.63\cdot10^{-5}$	$15.03\cdot10^{-5}\pm3.3\cdot10^{-6}$	0.999
	4.09	0.427	$14.53\cdot10^{-5}$		0.999

Table 3. Measured floc parameters in the presence of the tested additives

Additives / Parameter	Ca^{2+}	P 20,000	BPA 1,000	Magna Floc LT25
Floc size (10^{-6} m)	45 ± 5	59 ± 5	69 ± 6	127 ± 7
Floc porosity (%)	50.2 ± 1.1	55.0 ± 2.2	55.6 ± 2.3	57.8 ± 3.3
$-q_S\cdot10^{-5}$ ($kg\,kg^{-1}\,s^{-1}$)	5.92 ± 0.13	7.05 ± 0.33	8.90 ± 0.53	11.67 ± 0.33

Since the value obtained with Magna Floc LT25, was surprisingly high, we wondered if there was no influence of this polymer in the intrinsic metabolic activity of the yeast. In order to clarify any possible doubt, the influence of the additive in the glucose uptake rate of a non flocculating strain of S. cerevisiae was tested. Experiments performed in the described microreactor with S. cerevisiae sake in the presence of Ca^{2+} and of the additive Magna Floc gave the results presented in Table 2. The obtained straight lines were parallel and there was no difference in the specific glucose uptake rate of cells in the presence of Ca^{2+} or Magna Floc LT25.

Also, as expected, the specific glucose uptake rate for S. cerevisiae sake was larger than for the flocculating strain in the presence of Magna Floc LT25.

This confirms that the two fold increase in substrate consumption observed for the flocculating strain is due only to the physical effects caused by the additive, which most probably causes a reduction in floc internal diffusional limitations.

The next step was the determination of floc size and floc porosity (Table 3), since diffusional limitations are controlled by these two parameters. First, the evaluation of floc porosity was made, using a thermogravimetric method. It is clear from these determinations that, when compared to calcium, a statistically significant increase (*t*-test and 95% confidence interval) was observed in floc porosity in the presence of the tested polymeric additives.

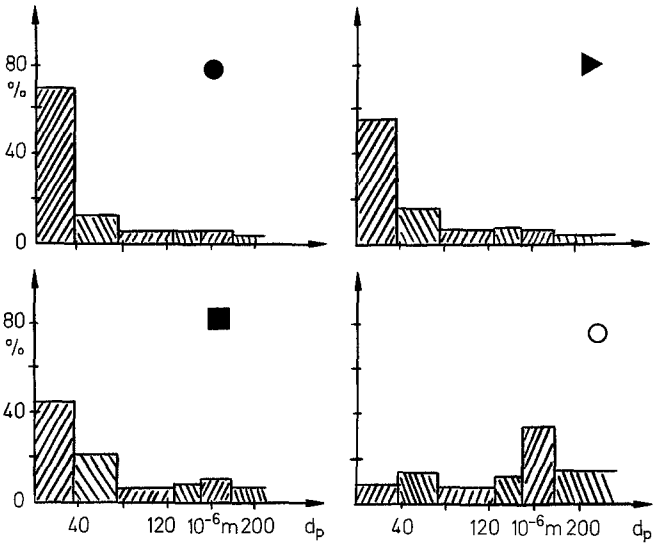

Fig. 3. Floc size distribution for the tested additives. ● – Ca^{2+}; ▶ – bis(polyoxyethylene-bis(amine)) 20,000; ■ – BPA 1,000; ○ – Magna Floc LT25

These values agree with reported data. For the same cells, Netto [23] reported a floc porosity of 50% in the presence of calcium and Teixeira and Mota [13] reported an identical value for flocs of K. marxianus, also in the presence of calcium. These authors also mentioned a 10% increase in floc porosity of K. marxianus in the presence of one of the tested additives – BPA 1,000.

This increase in porosity is not sufficient to explain such an increase in glucose consumption. Furthermore, the porosities for P 20,000, BPA 1,000 and Magna Floc LT25 being similar, there are significant differences in glucose consumption rates.

The floc size for S. cerevisiae flocs is the weighted average of the size distribution in the presence of the additives (Fig. 3). These values are a mean of six experimental determinations. It may be seen that the specific glucose uptake rate increases with the mean floc size, which disagrees with what should be expected in diffusion controlled processes.

The size distribution data, together with floc porosity determinations, indicated that some other factor must be responsible for the significant increase in glucose uptake rate. It must be reminded that a 2-fold increase in glucose uptake rate was observed in the presence of Magna Floc when comparing with calcium and that Magna Floc bound flocs are three times larger than Ca^{2+} bound flocs.

Therefore it is of fundamental importance to find a parameter that may constitute an explanation for the observed values of glucose consumption. For each additive, the distance δ between each individual cell in the floc was estimated. As a matter of fact, this parameter is a measure of the available flux area for glucose. To estimate this parameter, it was necessary to measure flocs specific gravity – *d*. The obtained calcium bound flocs specific gravity was 1.11. With this value, the specific gravity of the flocs bound by the tested

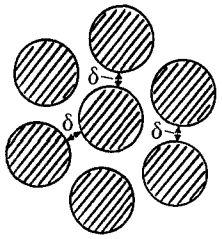

Fig. 4. Proposed model for yeasts floc packing

Table 4. Floc specific gravity and floc void volume for the tested additives

Additives / Parameter	Ca^{2+}	P 20,000	BPA 1,000	Magna Floc LT25
Floc specific gravity	1.11	1.10	1.10	1.09
Floc void volume (%)	55.7	60.5	61.2	63.0

additives was calculated. These values and the corresponding floc void volumes are presented in Table 4.

The additional following assumptions were made:

- Yeast cells are approximately spherical with a diameter of $6 \cdot 10^{-6}$ m.
- The distance between yeast cells in Ca^{2+} flocs is zero. In other words, this means that with S. cerevisiae cells the minimum floc void volume is 55.7%.
- The additive molecules are rigid and define the minimum distance δ between each individual cell in the flocs, according to the scheme (Fig. 4).
- The distance δ is the additive average length.
- The system formed by individual cells bound by an additive behaves, for packing considerations, like a set of spheres with a $(6+\delta) \cdot 10^{-6}$ m diameter, packed as if they were yeast cells bound by Ca^{2+} molecules with a diameter of $(6+\delta) \cdot 10^{-6}$ m.

The calculated distance between individual cells $\delta\,(10^{-6}\,\mathrm{m})$ in the flocs and specific glucose uptake rate $q_S\,(\mathrm{kg\,kg^{-1}\,s^{-1}})$ are correlated by the equation:

$$-q_S = 5.33 \cdot 10^{-5} + 1.42 \cdot 10^{-4} \cdot \delta \qquad (3)$$

with a c.c. of 0.90 and a percent mean deviation of 10.5%. This relation is plotted in Fig. 5.

Considering that the variation of floc size and floc void volume cannot explain the observed values of glucose uptake rate it may be advanced that the tested additives act on the reduction of glucose diffusional limitations mainly by increasing the intercellular distances in the flocs. Probably, a small increase in the void volume, may increase significantly the intercellular distances, thereby changing the available flux area of glucose or any other nutrient or product.

Although this approach seemed to be a good analysis of the available data, the relatively low coefficient of correlation led us to check, for different kinds of packing, how interparticle distance affects floc void volume.

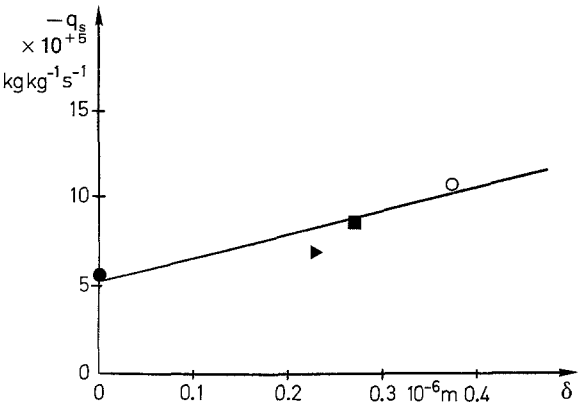

Fig. 5. Variation of specific glucose uptake rate q_s with floc intercellular distance δ for the tested additives. ● – Ca^{2+}; ▸ – bis(polyoxyethylene-bis(amine)) 20,000; ■ – BPA 1,000; ○ – Magna Floc LT25

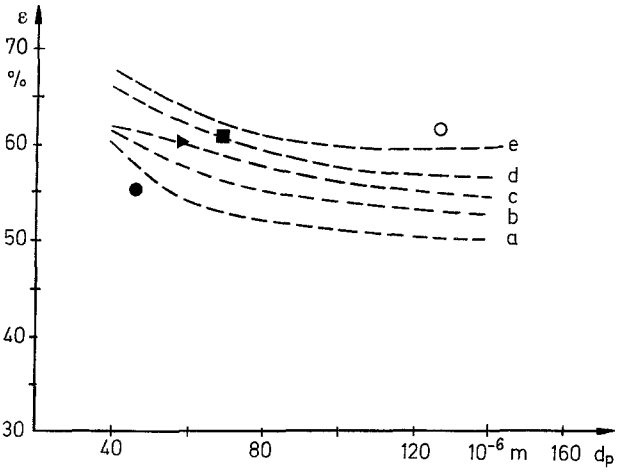

Fig. 6. Simulated floc void volume ε for different floc sizes d_p and different interparticle distances δ for cubic packing. a: $\delta = 0$; b: $\delta = 0.1$; c: $\delta = 0.2$; d: $\delta = 0.3$; e: $\delta = 0.4$ (10^{-6} m). ● – experimental values for Ca^{2+} bound flocs; ▸ – experimental values for P 20,000 bound flocs; ■ – experimental values for BPA 1,000 bound flocs; ○ – experimental values for Magna Floc LT25 bound flocs

There are four possible arrangements for monosized spherical particles [24]: the cubic, the orthorhombic, the tetragonal sphenoidal and the rhombohedral. Theoretically, when the interparticle distance is zero, the available void volumes are, respectively, 47.64%, 39.54%, 30.19% and 25.95%. Therefore we only considered the simulation of the cubic packing, since the theoretical values for the void volumes of the other packings are much lower than the experimental results.

For the cubic packing, the simulated floc void volumes for different floc sizes and different interparticle distances and the experimental values for floc size and floc void volume for the tested additives are presented in Fig. 6.

From this analysis, several conclusions can be drawn:

- The porosity increases as the interparticle distance increases.
- The packing arrangement for yeast cells flocs seems well characterized by the cubic packing. As a matter of fact, the calculated floc void volumes assuming this packing arrangement and the calculated interparticle distances are close to the experimental values.
- The presence of the additive may explain the observed porosity values, for yeast flocs.

This analysis reinforces the hypothesis that the available flux area on yeast cell flocs is controlled by the addition of charged polymers.

It may be concluded that flocculation additives can be used to reduce diffusional limitations in the flocs and so increase the observed reaction rate. Such an improvement is accomplished by increasing the available flux area for solutes, inside the flocs. Polymeric additives – anionic and cationic – act by controlling the distance between individual cells in yeast flocs. This hypothesis is confirmed by calculating the floc void volume for cubic packing. Anyway, this analysis should be enlarged to a wider range of additives with well known physical and chemical properties, namely, dimensions, structure and charge. Also, it is important to mention that both anionic and cationic additives may be used to reduce flocs diffusional limitations. Cationic additives may act by binding negatively charged yeast cell walls. Anionic additives, most likely, act by bridging calcium ions that are bound to the yeast cell walls.

The utilization of polymeric additives with the consequent increase in reaction rate may be of fundamental importance to obtain high productivity flocculating systems.

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